

Keywords: IRF-1; IFN- γ ; immune phenotype; NF- κ B; TNF- α ; nuclear translocation

IRF-1 responsiveness to IFN- γ predicts different cancer immune phenotypes

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Background: Several lines of evidence suggest a dichotomy between immune active and quiescent cancers, with the former associated with a good prognostic phenotype and better responsiveness to immunotherapy. Central to such dichotomy is the master regulator of the acute inflammatory process interferon regulatory factor (IRF)-1. However, it remains unknown whether the responsiveness of IRF-1 to cytokines is able to differentiate cancer immune phenotypes.

Methods: IRF-1 activation was measured in 15 melanoma cell lines at basal level and after treatment with IFN- γ , TNF- α and a combination of both. Microarray analysis was used to compare transcriptional patterns between cell lines characterised by high or low IRF-1 activation.

Results: We observed a strong positive correlation between IRF-1 activation at basal level and after IFN- γ and TNF- α treatment. Microarray demonstrated that three cell lines with low and three with high IRF-1 inducible translocation scores differed in the expression of 597 transcripts. Functional interpretation analysis showed mTOR and Wnt/ β -catenin as the top downregulated pathways in the cell lines with low inducible IRF-1 activation, suggesting that a low IRF-1 inducibility recapitulates a cancer phenotype already described in literature characterised by poor prognosis.

Conclusion: Our findings support the central role of IRF-1 in influencing different tumour phenotypes.

A dichotomy between immunologically active and quiescent cancer phenotypes has been recently demonstrated for several types of cancer (Wang *et al*, 2002, 2004; Galon *et al*, 2006; Leffers *et al*, 2009; Ascierto *et al*, 2012). Of particular interest, in colorectal cancer the type, density and location of immune cells have been shown to predict patient survival (Galon *et al*, 2006). Moreover, metastases from cutaneous melanoma have been previously shown to segregate into two different subclasses according to the

expression of genes annotated with immune function (Wang *et al*, 2002; Marincola *et al*, 2003). The recognition of the importance of the host immune system in controlling tumour progression have prompted several groups around the world to include immunological biomarkers in the conventional classification of tumours (Galon *et al*, 2012).

Central to the existence of the immune active phenotype in cancer is interferon regulatory factor (IRF)-1, the master regulator

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Revised 12 May 2013; accepted 23 May 2013;
published online 27 June 2013

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of the acute inflammatory process. Recent studies observed that the constitutive activation of the interferon (IFN)- γ /signal transducers and activators of transcription (STAT)-1/IRF-1 axis (Th1 phenotype) bears good prognostic connotation and predicts better responsiveness to anti-cancer therapy (Ascierto *et al*, 2011). IRF-1 is a nuclear transcription factor crucial to inflammation, immunity, cell proliferation and apoptosis (Kroger *et al*, 2002). Its synthesis is induced in response to IFN- γ (Taniguchi *et al*, 2001). The biological effects of IFN- γ are elicited through activation of intracellular molecular signaling networks, of which the best characterised is the JAK (Janus kinase)-STAT pathway. Conventional IFN- γ signaling follows the binding of IFN- γ to its cell surface receptor, which consists of two subunits IFNGRs (interferon gamma receptors)-1 and -2, and each molecule interacts with a member of the JAK family, which are non-receptor protein tyrosine kinases. JAKs phosphorylate STATs receptors and transcriptional co-activators leading to the downstream expression and activation of IRF-1 (Platanias, 2005). Although the relevance of IRF-1 to the immune biology of cancer is emerging, it remains unknown whether the responsiveness of IRF-1 to IFN- γ is able to point out different cancer behaviours. Here, we explored the significance of the cytokines-induced activation of IRF-1 in melanoma cell lines *in vitro* as a determinant of the immune behaviour of 15 melanoma cell lines. This question is relevant, because it may suggest that intrinsic characteristics of tumour cells that can be defined *in vitro* may be responsible for the responsiveness of cancer cells to signals produced by other cells, including immune cells, within the tumour microenvironment.

Although IRF-1 over expression in tumour lesions has been associated with better prognosis and likelihood of response to immunotherapy, such association is not absolute, suggesting that other factors are involved in the process. Several years ago, IRF-1 has been demonstrated to synergise with NF- κ B and assemble to form a higher-order nucleoprotein complex, the enhanceosome, a structure that modifies the local chromatin architecture and recruits the RNA polymerase II machinery to the promoter of several genes (Escalante *et al*, 1998; Panne, 2008). There is ample evidence that components of IFN- and NF- κ B-dependent signaling cross-interact to promote or repress gene expression by other pathways. Furthermore, it has been shown that TNF- α , a strong stimulator of NF- κ B, and IFN- γ are biologically related to each other. However, although TNF- α and IFN- γ have been shown to cooperate, the mechanisms behind this phenomenon are unknown. Here, we assessed whether IRF-1 and NF- κ B are concordantly activated in 15 melanoma cell lines, whether their concurrent activation is maintained after stimulation with IFN- γ , TNF- α and IFN- γ plus TNF- α , respectively, and whether it is mediated by their nuclear co-localisation.

Toward this goal, we used the ImageStream Flow Cytometry technique that permitted rapid and objective analysis of individual-cell IRF-1 nuclear translocation.

The present study shows a strong positive correlation between basal status of IRF-1 activation and its IFN- γ -induced activation, suggesting that IFN- γ has a strong effect towards IRF-1 nuclear translocation. This study also shows that the IFN- γ -inducibility of IRF-1 is able to point out different intrinsic properties of cancer cells. Moreover, we demonstrate that IRF-1 activation is additively induced by IFN- γ and TNF- α and that it is concurrently activated together with NF- κ B, although such co-activation is not due to a direct physical interaction of these two transcription factors.

MATERIALS AND METHODS

Melanoma cell culture. Melanoma cell lines were derived from metastatic melanoma lesions from patients treated at the Surgery

Branch, National Cancer Institute (NCI), Bethesda, MD, USA kindly donated by Dr. Steven A. Rosenberg. The cells received from Surgery Branch were studied after passage 3. Cells were cultured in bulk at 37 °C, in 5% CO₂ with RPMI 1640 medium (Gibco, Life Technologies, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (Cellgro, Mediatech, Inc. Manassas, VA, USA), 0.01% L-Glutamine Pen-Strep Solution (GPS 100 ×, Gemini Bio-Products, West Sacramento, CA, USA), 0.001% Ciprofloxacin (10 mg ml⁻¹) and 0.01% Fungizone Amphotericin B (250 μg ml⁻¹, Gibco). Confluent adherent cells were washed twice with phosphate-buffered saline 1X (PBS pH 7.4, Gibco) and detached by exposure to 0.2% Trypsin-EDTA (0.5%: 0.53 mM Solution, Gemini Bio-Products). The harvested cells were centrifuged to remove cell debris and re-suspended in fresh medium to a final concentration of 1 × 10⁷ cells ml⁻¹. Early-passage cultures were used for all experiments, and no clonal subselection was performed.

Aphidicolin-induced cell-cycle synchronisation. A stock solution of 1 mg ml⁻¹ aphidicolin from *Nigrospora sphaerica* (Sigma-Aldrich Co., St. Louis, MO, USA) was prepared in sterile dimethyl sulphoxide (DMSO, Sigma-Aldrich Co.) and stored in aliquots at -20 °C. Cells were seeded at a concentration of 1 × 10⁵ ml⁻¹ and, once attached, placed into medium containing 2.5 μg ml⁻¹ aphidicolin, which was an optimal reversible dose based on previous experiments (data not shown). After 24 h, cells were placed in a fresh medium.

In order to assess cell-cycle stage, 5-bromo-2-deoxyuridine (BrdU, Sigma-Aldrich Co.) was added to the cells to a final concentration of 10 μM. After 30-min incubation, cells were trypsinised and centrifuged at 3000 g for 5 min. Cells were then washed with PBS, fixed in ice-cold 70% ethanol and stored at 4 °C over night. Before staining, a 30-min incubation on ice with 0.1 M HCl containing 0.7% Triton X-100 (Sigma-Aldrich Co.) was performed. Cells were washed with 1 ml of 0.1 M sodium tetraborate (Sigma-Aldrich Co.), pH 8.5, to neutralise the acid, and re-suspended in PBS containing 0.5% Tween 20 (Sigma-Aldrich Co.). Cells were then incubated with anti-BrdU antibody (BD Biosciences, San Jose, CA, USA) (1:200) for 30 min, followed by 30 min with a 1:500 dilution of fluorescein (FITC)-conjugated secondary antibody. Cell nuclei were counterstained with 10 μg ml⁻¹ propidium iodide (PI, Sigma-Aldrich Co.), BrdU incorporation and total DNA content were then assayed using a FACSVantage SE cell sorter (BD Biosciences). The resulting data were processed and statistically analysed using CellQuest analysis software (BD Biosciences).

Immune stimulation. IRF-1 and NF- κ B translocation was activated by 1-h exposure to recombinant human IFN- γ (10 ng ml⁻¹) and recombinant human TNF- α (10 ng ml⁻¹) (R&D Systems, Inc., MN, USA), respectively. Concentration and time of exposure were chosen as optimal to achieve maximum translocation of the two transcription factors, according to preliminary experiments (data not shown). Following activation, cells were fixed in 4% (v/v) paraformaldehyde (PEA, Polysciences Inc, Warrington, PA, USA), permeabilised with 100% methanol and stained as outlined below.

ImageStream Flow Cytometry analysis. IRF-1 and the p65 subunit of NF- κ B were visualised by indirect immunofluorescence labeling. The primary mouse monoclonal anti-IRF-1 (clone B1, sc-137061) and rabbit polyclonal anti-NF- κ B p65 (ab7970) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Abcam (Cambridge, MA, USA), respectively. Fixed cells were incubated for 45 min at room temperature with both primary antibodies (1 μg per 10⁶ cells), washed twice with PBS and incubated for 45 min in the dark with both secondary antibodies (1 μg per 10⁶ cells) Alexa Fluor 647-conjugated F(ab')₂

fragment donkey anti-mouse IgG and Alexa Fluor 488-conjugated F(ab')₂ fragment donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA). After 30-min incubation with the secondary antibodies, 1 μ g DAPI per 10⁶ cells was added for the nuclear staining. Cells were finally washed twice with PBS and re-suspended in a PBS solution containing 0.1% sodium azide and 1% pen-strep-neomycin solution (Sigma-Aldrich Co.). Cells were then stored at 4 °C until analysis.

The basal status of IRF-1 activation was assessed first by immunofluorescence staining, which showed a different pattern of IRF-1 nuclear translocation among the cell lines (Supplementary Figure S1) and then by ImageStream analysis. For ImageStream analysis, a total of 2000–5000 events was collected from each sample. Data were acquired using INSPIRE and analysed by IDEAS software (Amnis Corp., Seattle, WA, USA). IRF-1 and NF- κ B nuclear translocation was quantitatively measured using similarity analysis on in-focus single cells, and expression levels were analysed as the median fluorescence intensity (George *et al*, 2006; Zuba-Surma *et al*, 2007).

Microarray analysis. Total RNA was isolated using miRNeasy minikit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. RNA quality and quantity was estimated using Nanodrop (Thermo Scientific, Barrington, IL, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). First- and second-strand cDNAs were synthesised from 300 ng of total RNA according to the manufacturer's instructions (Ambion WT Expression Kit, Affymetrix Inc., Santa Clara, CA, USA). cDNAs were fragmented, biotinylated and hybridised to the GeneChip Human Gene 1.0 ST Arrays (Affymetrix WT Terminal Labeling Kit). The arrays were washed and stained on a GeneChip Fluidics Station 450 (Affymetrix); scanning was carried out using the GeneChip Scanner 3000 and image analysis using the Affymetrix GeneChip Command Console Scan Control. Expression data were normalised, background-corrected and summarised using the RMA algorithm, <http://www.partek.com/>. Data were log-transformed (base 2) for subsequent statistical analysis.

Cluster analysis was performed using BRB-ArrayTools developed by the Biometric Research Branch, National Cancer Institute (<http://linus.nci.nih.gov/BRB-ArrayTools.html>) (Simon *et al*, 2007) or Partek Genomics Suite software (St Louis, MO, USA) as appropriate. Functional gene network analysis was performed using the Ingenuity Pathway Analysis system tools 3.0, which

transforms large data sets into a group of relevant networks containing direct and indirect relationships among genes based on known interactions in the literature (<http://www.ingenuity.com>).

Statistical analysis. Two-tailed Spearman correlation tests were applied to compare basal and IFN- γ -, TNF- α - and IFN- γ plus TNF- α -induced IRF-1 translocation and to compare IRF-1 and NF- κ B nuclear activation among the cell lines. A *P*-value ≤ 0.05 was considered statistically significant. Tests were performed using the Prism GraphPad software (GraphPad Software, Inc., La Jolla, CA, USA) and were all two-sided.

RESULTS

IRF-1 basal activation positively correlates with IFN- γ - and TNF- α -induced activation. In order to assess IRF-1 responsiveness to IFN- γ , we stimulated the 15 melanoma cell lines and evaluated the IRF-1 nuclear translocation by ImageStream IDEAS analysis software (George *et al*, 2006; Zuba-Surma *et al*, 2007). The cells were previously synchronised in early S-phase with aphidicolin and the efficiency of the synchronisation was assessed by the BrdU-based cell-cycle assay (Supplementary Figure S2). The basal IRF-1 nuclear translocation scores of the 15 cell lines are displayed in the Supplementary Table 1.

Our data indicate that the cell lines expressed significantly higher IRF-1 level in the nucleus after stimulation with IFN- γ ($P = 0.001$, Figure 1A). Moreover, the correlation of IRF-1 activation between controls and IFN- γ -stimulated cells was statistically significant ($P = 0.007$, $\rho = 0.65$, Figure 1B), suggesting that IFN- γ has a strong effect in inducing IRF-1 activation.

We next assessed whether IRF-1 activation induced by TNF- α and by IFN- γ plus TNF- α was correlated to its basal level as well. The correlation after TNF- α stimulation resulted even slightly stronger than the one induced by IFN- γ alone ($P = 0.007$, $\rho = 0.66$, Figure 1C), confirming the role of TNF- α as a strong inducer of IRF-1 nuclear translocation. However, the correlation of IRF-1 activation between controls and IFN- γ plus TNF- α -stimulated cells was not statistically significant although it maintained a positive trend ($P = 0.27$, $\rho = 0.30$, Figure 1D).

The correlation between basal IRF-1 and the delta of IRF-1 activation after stimulation with IFN- γ , TNF- α and IFN- γ plus

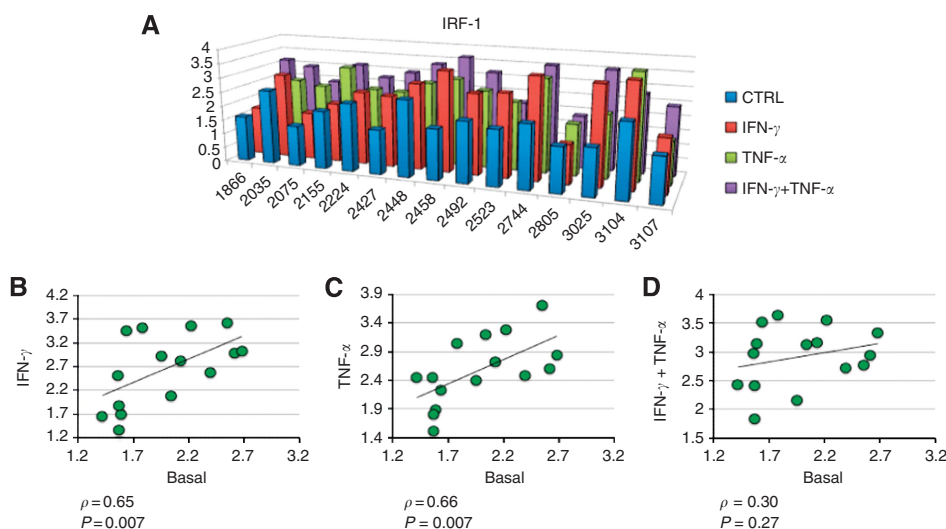


Figure 1. IRF-1 activation after cytokine stimulation. (A) IRF-1 nuclear translocation scores in controls, IFN- γ -, TNF- α - and IFN- γ plus TNF- α -stimulated cell lines. Correlation of IRF-1 nuclear translocation scores between basal level and after (B) IFN- γ , (C) TNF- α , and (D) IFN- γ plus TNF- α stimulation. ρ : Spearman correlation.

TNF- α did not reach statistical significance, suggesting that the IRF-1 activation was mainly dependent on the cytokine stimulation rather than on the basal level of IRF-1 activation (Supplementary Figure S3).

IRF-1-inducible activation predicts differential transcriptome behaviour in melanoma cell lines. To assess whether the responsiveness of IRF-1 activation to cytokine stimulation was able to distinguish different phenotypes in melanoma cell lines, we selected three cell lines with an overall low and three with an overall high inducible IRF-1 activation among the three experimental points (IFN- γ , TNF- α and IFN- γ plus TNF- α stimulation) and performed microarray analysis (Figure 2A). When comparing the three cell lines with low vs the three with high IRF-1-inducible translocation scores, we found 597 annotated transcripts whose expression was significantly different among the two groups ($P=0.05$, Supplementary Data). A self-organising map based on such transcripts completely discriminated the three cell lines with low from the three with high IRF-1-inducible activation (Figure 2B). Interestingly, when using these transcripts for functional interpretation analysis, we found mTOR and Wnt/ β -catenin as the top downregulated pathways in the cell lines with low inducible IRF-1 activation (Figures 2C and D, Supplementary Figures S4 and S5). Noteworthy, such pathways have been already shown to be associated with a poor phenotype in melanoma (Bittner *et al*, 2000; Spivey *et al*, 2012). These findings suggest that differential IRF-1 responsiveness to IFN- γ and TNF- α reflects a

differential behaviour of cell lines in terms of tumour aggressiveness and poor phenotype.

Quite interestingly, IL-24 was upregulated by up to 11-fold in cell lines with high inducible IRF-1. This is particularly intriguing as IL-24 has been shown to induce apoptosis in melanoma cell lines (Ekmekcioglu *et al*, 2008), thus its upregulation might reflect its potential synergism with IRF-1.

IRF-1 and NF- κ B are additively activated. We next evaluated whether IRF-1 and NF- κ B were mutually exclusively or concurrently activated in the 15 melanoma cell lines at basal level and after IFN- γ , TNF- α and IFN- γ plus TNF- α stimulations. Toward this goal, we used ImageStream Flow Cytometry analysis. Cells displaying different IRF-1 and NF- κ B subcellular localisation were first singularly visualised by ImageStream INSPIRE and then analysed with ImageStream IDEAS software, which allowed the assessment of population statistics of each single cell line (Figures 3A and B).

TNF- α alone and in combination with IFN- γ significantly increased the NF- κ B translocation score compared with the controls (Supplementary Figure S6).

When assessing the concurrent activation of IRF-1 and NF- κ B, we found that IRF-1 and NF- κ B nuclear translocation scores positively correlated in all the experimental conditions. However, such correlation reached a statistical significance only at basal level ($P=0.03$, $\rho=0.53$, Figure 3C) and after IFN- γ plus TNF- α stimulation ($P=0.02$, $\rho=0.60$, Figure 3D).

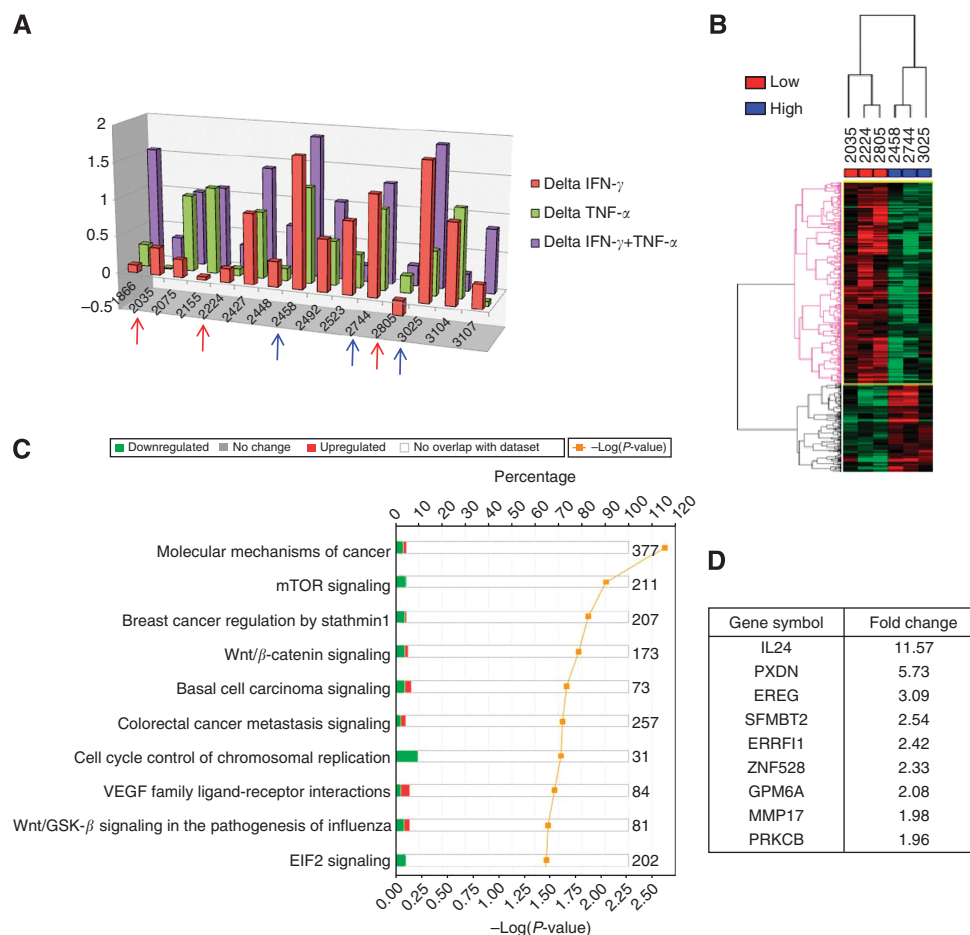


Figure 2. Delta IRF-1 activation after cytokine stimulation. (A) Bar graph showing the delta of IRF-1 activation scores in the 15 melanoma cell lines. The three cell lines with an overall low and the three with an overall high inducible IRF-1 activation are highlighted by the red and blue arrows, respectively. **(B)** Self-organising map of three low and three high inducible IRF-1 expressing cell lines. **(C)** Functional interpretation analysis of the transcripts differentially expressed between the two groups of cell lines. **(D)** Top transcripts differentially expressed between high and low IRF-1 cell lines.

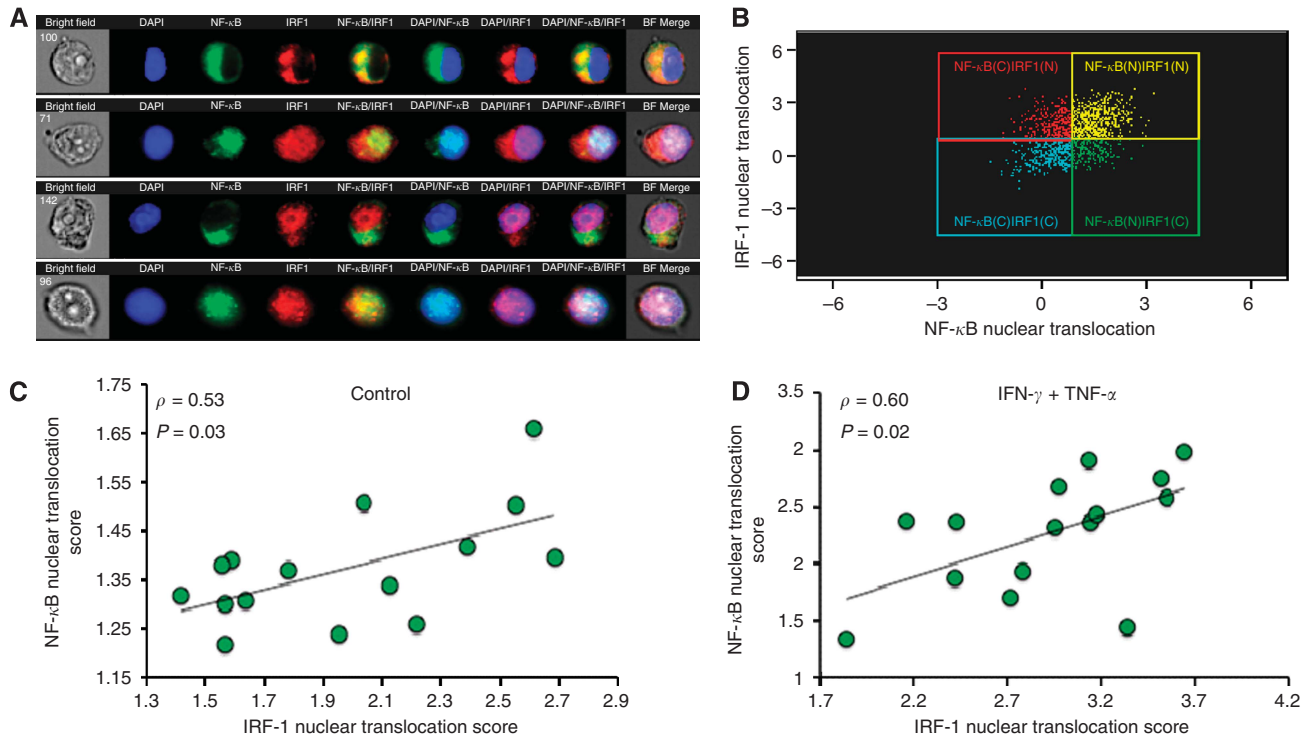


Figure 3. NF- κ B and IRF-1 activation. **(A)** Single-cell images showing different NF- κ B and IRF-1 subcellular distribution. From the top to the bottom: NF- κ B and IRF-1 predominantly located in the cytoplasm; NF- κ B predominantly translocated to the nucleus and IRF-1 predominantly localised in the cytoplasm; NF- κ B predominantly localised in the cytoplasm and IRF-1 predominantly translocated to the nucleus; and both NF- κ B and IRF-1 predominantly translocated to the nucleus. **(B)** Population statistics of the IRF-1 and NF- κ B nuclear translocation of the cell line # 2155. **(C)** Correlation between NF- κ B and IRF-1 nuclear translocation scores in controls and **(D)** in IFN- γ plus TNF- α -stimulated cell lines. ρ : Spearman correlation.

In addition, given the hypothesis that if IRF-1 and NF- κ B interact with each other they should both co-localise to the nucleus, we tested their nuclear co-localisation score as well. Contrary to our hypothesis, IRF-1 and NF- κ B did not significantly co-localise in any experimental condition. Thus, although their activation resulted in an additive effect, it was not mediated by their physical interaction, at least under our experimental conditions.

DISCUSSION

Several recent studies have shown that a neoplastic phenotype enriched in immune infiltrates correlates with good prognosis and enhanced likelihood to respond to immunotherapy (Galon *et al*, 2006; Ascierto *et al*, 2011, 2012). Our group observed that IFN- γ has a central role in the immunologically active cancer phenotype characterised by enhanced expression of IRF-1. This, in turn, correlates with the activation of transcripts associated with improved survival in cancer (Galon *et al*, 2006; Ascierto *et al*, 2012; Spivey *et al*, 2012). However, the mechanisms leading to the activation of IRF-1 in cancer are still unknown. Assessing the cytokine-mediated IRF-1 activation may help in understanding the intrinsic mechanisms underlying differential immune behaviour of cancer cells.

IFN- γ is a pleiotropic cytokine that induces gene expression through activation by tyrosine and serine phosphorylation of components of the JAK-STAT pathway (Darnell *et al*, 1994; Darnell, 1997; Stark *et al*, 1998), by binding to gamma-activated sequence (Stark *et al*, 1998; Shultz *et al*, 2009) or to IFN-stimulated response elements in the promoters of the IFN-stimulated genes. In this report, we assessed IRF-1 activation by using the

ImageStream Flow Cytometry approach. Knowledge of the cellular localisation of a given transcription factor can provide insights about its activation; indeed, when the transcription factor is primarily sequestered in the cytosol, its ability to regulate gene transcription could be inhibited. We used the ImageStream nuclear localisation score to assess IRF-1 activation (George *et al*, 2006).

After treatment with IFN- γ and TNF- α , we observed a significant increase of IRF-1 nuclear translocation. The induction of IRF-1 activation after cytokine stimulation was a primary response that did not require protein synthesis, as it occurred within 1 h upon stimulation. Thus, even though several reports have shown that the effect of IFN- γ and TNF- α occur at the transcription and protein level (Lamb *et al*, 1995; Pine, 1997; Lee *et al*, 2000), our data suggest that the IRF-1 activation may be mediated by mechanisms, which do not require *de novo* protein synthesis.

Given that different cell lines have different amplitude of response to cytokine stimulation, we asked whether the inducibility of IRF-1 by IFN- γ and TNF- α was able to distinguish a parallel different behaviour of cancer cells. Toward this aim, we measured the difference of nuclear translocation score between controls and stimulated cells and further selected three cell lines with low and three cell lines with high delta of IRF-1 nuclear translocation among the three experimental points (IFN- γ , TNF- α and IFN- γ plus TNF- α stimulation). When comparing the basal transcriptome of these cell lines, we found 597 annotated transcripts significantly different between the two groups. Most interestingly, functional interpretation of such transcripts revealed mTOR and Wnt/ β -signaling among the mostly downregulated pathways in cell lines with high IRF-1 responsiveness to IFN- γ and TNF- α . This is in perfect concordance to previous studies, which showed the existence of at least two immune phenotypes in melanoma: a Th17

phenotype associated with overexpression of components of Wnt and mTOR pathways, enhanced cellular motility, a more undifferentiated status and poor prognosis, and a Th1 immune phenotype associated with the expression of melanocytic lineage-specific transcripts, a more differentiated status and better prognosis (Wang *et al*, 2002; Spivey *et al*, 2012). The latter has been shown to centre around the activation of IRF-1 and to be characterised by a classic IFN-signature. Thus, the differential IRF-1 inducibility reflects a different cancer behaviour that has already been described in literature.

When assessing the activation of both IRF-1 and NF- κ B, we found the two transcription factors to be concurrently activated. Previous studies on human cell lines have demonstrated that TNF- α and IFNs co-regulate the expression of inflammation-associated genes such as MHC class I, intracellular cell adhesion molecule 1 and vascular cell adhesion molecule-1 in a synergistic manner (Doukas & Pober, 1990; Blanchet *et al*, 1991; Jahnke & Johnson, 1994; Johnson & Pober, 1994; Drew *et al*, 1995). However, when testing the co-localisation of the two transcription factors, we were not able to demonstrate their physical interaction, not even after cytokine stimulation. This suggests that the activation of the two transcription factors may determine the same effects; moreover, the fact that the co-localisation was never significant in all the experimental conditions suggests a mutual exclusively activation of IRF-1 and NF- κ B at a cell level. Thus, it is likely that IRF-1 and NF- κ B are activated in an additive rather than in a synergistic manner under our experimental system. Testing this idea with a variety of experimental models of melanoma is warranted to determine whether IRF-1 and NF- κ B interact with each other or act independently both at basal level and after IFN- γ and TNF- α stimulation. Moreover, we cannot exclude the possibility that NF- κ B p50 and/or NF- κ B p52 expression are activated and may compensate for any loss of NF- κ B activity (Cogswell *et al*, 2000).

In conclusion, our findings suggest that the responsiveness of IRF-1 to the immune cytokines IFN- γ and TNF- α is able to point out two different cancer phenotypes and support the central role of such transcription factor in influencing different tumour behaviours.

ACKNOWLEDGEMENTS

This study was financed by the National Institutes of Health; all NIH affiliated authors performed this work as part of their official duties. DB's scholarship was supported by the Conquer Cancer Foundation of the American Society of Clinical Oncology (2011 Young Investigator Award). DM's scholarship was supported by the Master and Back Program of Regione Autonoma della Sardegna, Italy.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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